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The Enigmatic Esx Proteins: Looking Beyond Mycobacteria

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1 **Abstract**

2 Bacteria export proteins across membranes using a range of transport
3 machineries. Type VII secretion systems (T7SSs), originally described in
4 mycobacteria, are now known to be widespread across diverse bacterial
5 phyla. Recent studies have characterized secretion components and
6 mechanisms of type VII secretion in pathogenic and environmental bacteria. A
7 variety of functions have been attributed to T7SS substrates, including
8 interactions with eukaryotes and with other bacteria. Here, we evaluate the
9 growing body of knowledge on T7SSs, with focus on the non-mycobacterial
10 systems, reviewing their phylogenetic distribution, structure and function in
11 diverse settings.
12

1 **Type VII Secretion: Discovery in Mycobacteria**

2 Bacteria possess a range of transport mechanisms that export proteins across
3 the cell envelope. Bacterial secretion systems that mediate protein
4 translocation across the bi-layered Gram-negative cell envelope have been
5 classified into six numbered types, Types I to VI [1]. In monoderm Gram-
6 positive bacteria, the Sec, Tat and flagellar secretion systems were initially
7 thought to account for all protein export, until the discovery of a new
8 specialised secretion system, ESX-1, in *Mycobacterium tuberculosis*. This
9 system first generated attention because it is responsible for the export of an
10 abundant immunogenic protein, ESAT-6 (early secreted antigen target 6; also
11 called EsxA) [2-4]. Additional substrates and components of ESX-1 have
12 since been identified and characterized, including multiple EccC family
13 ATPases [5], leading to the development of models that incorporate structure,
14 function and regulation of this complex secretion apparatus [6, 7]. In addition
15 to ESX-1, *M. tuberculosis* possesses paralogous ESX systems, ESX-2 to
16 ESX-5, which have broadly similar features [5, 8]. The ESX-1 system plays an
17 important role in *M. tuberculosis* virulence and a number of ESX-1 effectors
18 interfere in host cellular and immune functions, primarily by modulating host
19 macrophage and T cell responses [9]. However, the precise evolutionary and
20 functional context for these activities remains uncertain.

21

22 The numerical nomenclature for secretion systems was initially restricted to
23 Gram-negative bacteria. However, as it became clear that the mycobacterial
24 cell envelope also possessed a diderm structure analogous to the Gram-
25 negative cell wall, the nomenclature was extended to incorporate the

1 mycobacterial ESX systems, which were renamed type VII secretion systems
2 (T7SSs) [10].

3

4 Shortly after the discovery of type VII secretion in mycobacteria, genome
5 analyses suggested the existence of similar systems in diverse groups of
6 Gram-positive bacteria [11]. However, these T7SSs appeared to be less
7 complex than their mycobacterial counterparts, each with just a single
8 ATPase from the EccC family that contains several FtsK/SpoIIIE domains,
9 together with a handful of secretion substrates. These simpler systems were
10 given the name type VIIb secretion systems to distinguish them from their
11 more complex mycobacterial relatives [12, 13]. Mycobacterial type VII
12 secretion has been reviewed extensively elsewhere [5, 13-15]. Here, we will
13 focus instead on type VIIb secretion systems, summarising new
14 developments in their phylogenetic distribution, structure and function.

15

16 **Type VII Secretion Occurs Widely Outside the Mycobacteria**

17 Analysis of the *M. tuberculosis* genome showed that EsxA is a member of a
18 family of paralogous proteins [16]. A small-scale comparative genome
19 analysis revealed that gene clusters encoding *esxA* homologues were also
20 found in several close relatives of *M. tuberculosis* within the Actinobacteria,
21 including *Corynebacterium diphtheriae* and *Streptomyces coelicolor* [8].

22 Predicted proteins representing EsxA homologues were subsequently
23 identified in several members of the other predominantly Gram-positive
24 phylum, the Firmicutes, including bacterial species with roles as pathogens,
25 model organisms or in biotechnology, such as *Staphylococcus aureus*,

1 *Bacillus anthracis*, *Streptococcus agalactiae*, *Bacillus subtilis* (Figure 1),
2 *Rhodococcus equi*, and *Clostridium acetobutylicum* [11, 17-20]. As sequence
3 conservation is centered on a WXG motif, and most of these EsxA homologs
4 were around 100 amino acids in length, the term “WXG100 proteins” was
5 coined to describe the family. In the Firmicutes, genes for WXG100 proteins
6 were located in gene clusters that also encoded a conserved ATPase, which
7 contained several FtsK/SpoIIIE domains and which was predicted to power
8 type VII secretion.

9

10 In a later study, Jang *et al.* showed that an uncharacterized protein, Hp0062,
11 from the Gram-negative pathogen, *Helicobacter pylori*, shared unexpected but
12 unequivocal structural homology with WXG100 proteins from *M. tuberculosis*
13 and *S. aureus* [21]. Subsequently, Sutcliffe examined the phylogenetic
14 distribution of the WXG100 proteins and reported their occurrence in four
15 phyla with a Gram-negative or diderm cell architecture, the Proteobacteria,
16 the Cyanobacteria, Lentisphaerae, Verrucomicrobiae as well as in the
17 monoderm phylum, Chloroflexi [22].

18

19 An up-to-date phylogenetic survey (Table 1) reveals the presence of WXG100
20 proteins in several additional bacterial phyla or sub-phyla (Table 1), including
21 all five sub-divisions of the Proteobacteria (incorporating several important
22 pathogenic species including *Pseudomonas aeruginosa*, *Vibrio cholerae* and
23 *Stenotrophomonas maltophilia*) together with the Fusobacteria, the
24 Planctomycetes and the Tenericutes.

1 Scrutiny of the entry for the WXG100 domain in the PFAM database
2 (PF06013) provides a ready guide to the various protein architectures that
3 house this feature (<http://pfam.xfam.org/family/PF06013#tabview=tab1>). In the
4 majority (>96%) of cases, this domain occurs in a simple one-domain
5 architecture, where the WXG100 domain occupies the entire protein.
6 However, there are now several examples of this domain within a multi-
7 domain protein, typically linked to an enzymatic domain. Most of these
8 domains are linked to toxin-antitoxin systems (e.g. PT-TG, Toxin-deaminase,
9 Colicin-DNase, Tox-HNH-EHHH, DUF2974, CHAP, Ntox44, and Ntox28). In
10 many cases these domains appear to be important participants in conflicts
11 between the cells that carry them and other cells. However, some of these
12 enzymatic domains may play other roles: for example, the lytic
13 transglycosylase activity of the PT-TG domain cleaves the cell wall
14 peptidoglycan making space for the assembly of membrane spanning
15 structures such as secretion systems and flagella [23]. Colicins and pyocins
16 [24, 25] contain HNH domains with DNase activities, acting specifically
17 against *Escherichia coli* and *P. aeruginosa* cells respectively, eliciting cell
18 death through random cleavage of chromosomal DNA [26]. The targets of
19 these systems are diverse including action against eukaryotic hosts, against
20 other bacterial species and against members of the same bacterial species
21 fighting for a common niche [25, 27-31]. Additional evidence for a link
22 between T7SSs and toxin-antitoxin systems comes from a wide-ranging
23 bioinformatics survey that reports fusions of WXG100 domains to toxin
24 domains and the presence of toxin and immunity proteins in many T7SS gene
25 clusters [27].

1

2 Interestingly, a recent analysis from Das *et al.* [32] revealed an association of
3 the putative EsxA homologue, HP_0062 [21], with an FtsK/SpoIIIE ATPase in
4 at least 21 complete *H. pylori* genomes. Besides the typical WXG proteins,
5 the cluster contains HP_0063, which has an HNH/colicin domain [27], as well
6 as a protein (HP_0064) that belongs to the SUKH superfamily prototyped by
7 the *Saccharomyces cerevisiae* protein Smi1/Knr4, which is responsible for
8 conferring resistance to the killer toxin produced by competing yeast (Figure
9 1). Nevertheless, experimental evidence is required to corroborate the
10 suggestions of putative T7SS from *H. pylori* and other Gram-negative
11 bacteria.

12

13 The WXG100 proteins have been classified into three subfamilies based on
14 their similarity to the heterodimeric mycobacterial proteins EsxA (ESAT-6) or
15 EsxB (CFP-10), which are encoded in bicistronic operons [17, 33], or to the
16 homodimeric non-mycobacterial type VIIb effector EsxA, which is typically
17 encoded in a monocistronic operon [17, 34, 35]. A key conserved feature
18 across the WXG100 protein families is a C-terminus consensus sequence
19 HxxxD/ExhxxxxH, where 'H' is a highly conserved hydrophobic residue and 'h'
20 is a less conserved hydrophobic residue [17]. In mycobacteria, a YxxxD/E
21 motif is the C-terminal secretion signal required for general targeting of type
22 VII substrates, including WXG100 proteins, and for the modulation of
23 substrate binding and activity of a central ATPase [6, 36, 37]. Similarly, the C-
24 terminal tail is important for secretion of type VIIb effectors in *S. aureus* and *B.*
25 *subtilis* [35, 38, 39]. In *B. subtilis*, substrate export requires a composite

1 secretion signal formed by both the WXG100 and C-terminal motifs, each
2 contributed by individual subunits of the substrate dimer [35]. Given the
3 conservation of these motifs across type VII substrates, they likely play a
4 crucial role in controlling type VIIb secretion [6].

5

6 Despite the now-abundant evidence from sequence-based analyses that type
7 VII secretion occurs outside the mycobacteria, type VIIb secretion systems
8 have been analysed experimentally only in a handful of bacterial genera,
9 including *Staphylococcus* and *Bacillus*.

10

11 ***The Staphylococcus aureus T7SS***

12 Among the type VIIb secretion systems, the best characterized is that of the
13 opportunistic human pathogen, *S. aureus*. The system, termed Ess (for
14 ESAT-6 secretion system), is required for virulence in murine abscess,
15 pneumonia and skin infection models [40-42] and is linked to the
16 establishment of persistent infection [40, 43]. The *ess* genes showed high
17 levels of upregulation during chronic *S. aureus* infection in cystic fibrosis
18 patients [44], suggesting a similar role in persistent infection in humans.

19

20 The Ess machinery comprises six core components [40, 41] (Figure 2),
21 including one extracellular protein, one cytoplasmic protein and four
22 membrane proteins. The most abundant component is EsxA, a secreted
23 WXG100 protein that forms a homodimer [34, 41, 45]. EsxA displays a high
24 degree of sequence conservation across *S. aureus* strains [45] and is
25 required for the secretion of other Ess substrates [38, 40, 41], strongly

1 implicating it as a core extracellular component. The second soluble protein is
2 EsaB, which harbours a ubiquitin-like fold [46] and appears to regulate the
3 Ess machinery [41, 43].

4

5 The four key membrane proteins include EssA, EssB, EssC and EsaA. While
6 EssA is required for substrate secretion, the requirement for EsaA appears to
7 be strain-dependent [41, 43]. X-ray structures are available for soluble
8 domains of EssB [47, 48] and EssC [6, 49]. EssB, has a cytoplasmic domain
9 with a pseudokinase fold, but it lacks ATP-binding signature motifs and is
10 unable to bind ATP-analogues [47].

11

12 EssC, the predicted FtsK/SpoIIIE-like motor protein, contains three C-terminal
13 P-loop ATPase domains (D1-D3). Deletion analysis suggests that all three are
14 essential for secretion [49]. Similarly, a transposon insertion in the N-terminal
15 region of D1 abolishes EsxA and EsxB secretion, but curiously, transposon
16 mutants with insertions in the last two domains remain capable of secretion
17 [40]. The reason for this discrepancy is not clear.

18

19 Proteins of the FtsK/SpoIIIE family, including EccC₁, are known to form
20 hexamers [6]. Although no structures have yet been obtained for the *S.*
21 *aureus* EssC ATPase domains, homology modelling has provided fresh
22 insights into this phenomenon. X-ray structures of the C-terminal P-loop
23 domains (D2 and D3) have been obtained from the EccC homologue from
24 *Geobacillus thermodenitrificans*. These structures show that D2 has ATP
25 bound whereas D3 exists in two conformations; ATP binding is occluded in

1 one of these [6, 49]. Modelling the X-ray structure of the C-terminal domains
2 of the *G. thermodentrificans* EssC on to the known hexameric structure of
3 FtsK suggests that EssC possesses an internal pore (diameter~30 Å) wide
4 enough to accommodate EsxA dimers [49].
5
6 EssC proteins from type VIIb secretion systems have tandem forkhead-
7 associated (FHA) domains at their N-termini, unlike the related mycobacterial
8 EccC proteins [49, 50]. FHA domains typically bind phospho-threonine
9 peptides and are known to mediate phosphorylation-dependent protein–
10 protein interactions [51]. However, no function has yet been assigned to the
11 FHA domains in EssC, apart from contributing to overall protein stability [49].
12 *In vivo* crosslinking analysis indicated that Ess membrane proteins homo-
13 multimerise [52] Although multimerisation of *Thermomonospora curvata* EccC
14 is controlled by substrate interactions, EssC multimerisation was observed in
15 a strain of *S. aureus* lacking core Ess components and substrates.[52]. Thus,
16 even if these ATPases show significant sequence homology, there appear to
17 be differences in the control of their assembly and activity.
18
19 Ess secretion has been studied in several *S. aureus* strains, including
20 Newman [40, 43, 53], USA300 [38], RN6390 and COL [41]. Genomic analysis
21 shows that the organisation of the *ess* loci in these strains is highly similar
22 although there is some transcriptional heterogeneity between the strains [41].
23 Transcription of *esxA*, the first gene of the cluster, is under control of the
24 alternative sigma factor σ^B [54]. EsxA is required for bacterial virulence and
25 was recently shown to suppress host cell apoptosis during intracellular

1 infection [39, 41, 43]. In addition to EsxA, four substrates of the secretion
2 system have been described: EsxB, EsxC, EsxD and EsaD.

3

4 EsaD is the only substrate to which a function has been assigned. Initial
5 analysis of an *esaD* mutant strain [32] indicated that EsaD was an accessory
6 factor required for the secretion of other Ess substrates. However, more
7 recently it has been shown that EsaD is a large type VII-secreted anti-
8 bacterial toxin, with a nuclease domain at its C-terminus that targets the DNA
9 of sensitive strains of *S. aureus* [31]. Two further *ess*-encoded proteins are
10 essential for EsaD biogenesis. EsaG is an EsaD anti-toxin, which binds to the
11 nuclease domain of EsaD, protecting the producing cell from self-intoxication.
12 Introduction of EsaG into a sensitive *S. aureus* strain offers significant
13 protection against EsaD-mediated killing. A second protein, EsaE, binds to the
14 non-nuclease part of EsaD and appears to target the EsaDG complex to the
15 secretion machinery [31], analogous to the chaperone EspG that interacts
16 with large PE/PPE substrate proteins in pathogenic mycobacteria [55, 56].
17 EsaG is probably stripped from EsaD during secretion as it is not detected in
18 the supernatant, although co-secretion of EsaE with EsaD has been noted
19 [31].

20

21 EsxB, EsxC and EsxD are all small secretion substrates, around 100 amino
22 acids in length, but only EsxB belongs to the WXG100 family [38, 40, 43]. The
23 functions of these proteins are not clear. The genes encoding EsxB and EsxC
24 are required for virulence in a mouse abscess model. However, as deletion of

1 these genes affects secretion and/or stability of other substrates, it is not clear
2 whether their requirement for virulence is direct or indirect [40, 43].

3
4 Analysis of 153 *S. aureus* genome sequences revealed that the *ess* locus falls
5 into four distinct clusters [45], three of which do not encode EsxB, EsxC, EsxD
6 or EsaD. Instead, each of these has a distinct set of genes downstream of the
7 core *ess* genes, indicative of discrete repertoires of substrates. The complete
8 repertoire of Ess-secretion substrates in the *S. aureus* pan-genome remains
9 to be elucidated, but it is likely that substrates will include further antibacterial
10 toxins and proteins that contribute to virulence.

12 ***Type VII secretion in Bacillus***

13 Type VII systems are present in both pathogenic and non-pathogenic species
14 of *Bacillus*. In the model organism *B. subtilis*, the core functional components
15 of an ESX secretion system are encoded by the *yuk/yue* locus that contains
16 *yukE*, *yukD*, *yukC*, *yukBA*, *yueB* and *yueC* (Figure 1) [57, 58]. YukBA is an
17 FtsK/SpoIIIE family ATPase that mediates the export of YukE, a member of
18 the WVG100 protein superfamily. YukE secretion and stability is dependent
19 on phosphorylated DegU, the response regulator of the DegU-DegP two
20 component system [57]. YukD is a small ubiquitin-like protein with a very short
21 C-terminal tail [46]. YukD is homologous to the *S. aureus* EsaB, which is
22 essential for secretion in some *S. aureus* strains [41, 43], while YukC is
23 similar to the *S. aureus* structural protein EssB [59]. YueB is a surface protein
24 that has been reported to accumulate at the bacterial poles, as observed for
25 the mycobacterial ESX-1 proteins [60, 61]. YueB is essential for phage SPP1

1 binding and the *yuk* operon, particularly YukE, contributes to phage infection
2 [59]. Recently, YueB was also implicated in conjugative DNA transfer [62].
3
4 Interestingly, unlike other type VII systems, YukE appears to be the only
5 substrate associated with the *Bacillus* system. Although a second potential
6 WXG100 substrate YfjA was identified in *B. subtilis* genome [10], there is no
7 evidence that the associated protein is exported in a YukBA-dependent
8 fashion [57, 58]. Utilizing this simple *Bacillus* system Sysoeva *et al* [35]
9 demonstrated that YukE was secreted as a homodimer, and that the WXG100
10 motif and a C-terminal sequence are required for its secretion. Intriguingly,
11 YukE was secreted as an intact dimeric complex and a bipartite signal formed
12 by the two motifs, each contributed by individual subunits of the dimer, was
13 required for its export [35]. As these motifs are typically present in type VII
14 substrates, this novel mechanism of substrate recognition and protein
15 translocation may be conserved across other type VII systems.
16
17 The ATPase domain requirements for substrate translocation in *Bacillus* were
18 recently dissected in a study of domain-specific nucleotide binding and
19 hydrolysis [63]. Only one of the three ATPase domains of YukBA was
20 required for secretion in *Bacillus*, similar to that observed in ATPases from
21 some *S. aureus* strains [40]. This is similar to the mycobacterial EccC
22 ATPases such as the EccC₅, where only ATP binding to the D1 domain is
23 necessary for secretion [64], but in contrast to the mycobacterial split ATPase
24 EccC₁, where nucleotide binding is required at all three domains [6, 63].
25

1 In the pathogen *B. anthracis*, six WXG100 proteins were discovered by
2 genome analysis: EsxB, EsxL, EsxP, EsxQ, EsxV and EsxW (Figure 1) [65].
3 EsxB and EsxW have been detected from culture filtrates *in vitro*, while EsxW,
4 EsxB and EsxP are expressed only during guinea pig infection [65]. It is
5 possible that *B. anthracis* Esx substrates are induced only under specific yet-
6 to-be-identified conditions.

7

8 As with *S. aureus*, an FtsK SpoIIIE-type ATPase, designated *B. anthracis*
9 EssC is required for EsxB secretion. Deletion of EsxB specifically affected
10 secretion of EsxW, and yeast-2-hybrid analysis showed that the two proteins
11 interact, suggesting that EsxB is required for the stability and secretion of
12 EsxW (although not the other way around) [65]. The crystal structure of EsxB
13 revealed a helix-loop-helix hairpin, which allows the protein to associate into
14 two distinct helical bundles [66]. This structural duality may enable formation
15 of homo and hetero-oligomeric helix bundles with other Esx substrates,
16 supporting the idea that EsxB could serve as an adaptor for secretion.

17

18 **Type VII Effectors and Host-Pathogen Interactions**

19 Type VII effectors from bacteria with intracellular lifestyles, particularly
20 mycobacteria, are adept at modulating host cell pathways. T7SS substrates
21 can interfere with or induce cellular functions in numerous ways, enabling the
22 bacterium to survive within the host [15] (Figure 3, Table 2).

23

24 **Host Cell Modulation**

1 The ESX-1 locus was originally discovered because loss of the locus was
2 associated with loss of virulence in the attenuated vaccine strain BCG [67].
3 ESX-1 effectors are required for the virulence of *M. tuberculosis* and impact
4 on several host functions including apoptosis, necrosis, induction of
5 interferons, cytolysis and granuloma formation [68-70]. EsxA directly interacts
6 with host cell membranes causing host cell lysis and phagosomal rupture,
7 which then results in the translocation of bacterial DNA and initiation of
8 cytosolic surveillance pathways leading to ubiquitination, autophagy and
9 interferon responses [71-73] (Figure 3).

10

11 In the facultatively intracellular pathogen *S. aureus*, Ess is required for
12 virulence and bacterial persistence during infection [40, 41, 43]. Interestingly,
13 EsxA delays host cell apoptosis, and EsxA and EsxB together facilitate
14 release of intracellular *S. aureus* from human epithelial cells [39]. While the
15 cellular mechanisms underlying the modulation of cell death need further
16 investigation and there are probably differences in the pathways targeted, it is
17 intriguing that Esx proteins from two diverse pathogens, *S. aureus* and *M.*
18 *tuberculosis* have been recruited to exploit the host cell as a niche for survival.
19 By contrast, the ESAT-6 homologue, EsxA, from the intracellular pathogen,
20 *Listeria monocytogenes* appears to play no role in intracellular survival or
21 virulence [74]. Furthermore, a recent study reported that *L. monocytogenes*
22 lacking EsxA demonstrated increased virulence, suggesting a detrimental
23 effect of ESX-1 during infection [75].

24

25 ***Immune Modulation***

1 The effectors of ESX-1, ESX-3 and ESX-5 are potent activators of CD4+ and
2 CD8+T cells [76-78]. Type VII effectors such as EsxA and EsxH have become
3 prime anti-tuberculosis vaccine candidates because of their strong
4 immunogenic properties [78]. Similarly, the EsxA and EsxB proteins from *S.*
5 *aureus* are being considered as vaccine candidates because they induce a
6 strong protective antibody response in murine infection models [79]. However,
7 it is not clear whether staphylococcal Esx proteins induce cell-mediated
8 immune responses.

9

10 **Bacterial Functions of Type VII Systems**

11 The conservation of the T7SS across diverse phyla indicates that these
12 proteins may play important roles in bacteria. The type VII effectors have
13 been associated with a number of fundamental bacterial pathways, in both
14 pathogenic and non-pathogenic species (Table 2, Figure 3).

15

16 ***DNA Transfer***

17 Soon after its discovery, ESX-1 was shown to be essential for conjugative
18 DNA transfer in the environmental mycobacterium, *Mycobacterium smegmatis*
19 [80, 81]. ESX-1 effectors are thought to influence DNA transfer by
20 chaperoning proteins specific to donors or recipients, thereby inhibiting or
21 promoting donor-recipient cell contact. Remarkably, ESX-1 was recently
22 shown to encode a switch in mating identity from recipient to donor in
23 transconjugants. The mating identity (*mid*) locus was mapped within the *esx-1*
24 (*Ms0069-0071 and Ms0076-0078*) and was shown to have orthologs in other
25 environmental mycobacteria [82]. ESX-1 may be required for DNA transfer

1 simply because it mediates secretion of the Mid proteins which dictate the
2 mating identity. In *B. subtilis*, YueB, is also known to play a role in conjugative
3 transfer of the plasmid pLS20. However, the core T7SS ATPase, YukBA, was
4 not directly involved in DNA transfer [62]. The mechanism of YueB-mediated
5 plasmid transfer remains unknown.

6

7 ***Metal Uptake***

8 The mycobacterial ESX-3 T7SS has been implicated in the uptake of metal
9 ions [83, 84]. In *M. smegmatis* and *M. tuberculosis*, expression of ESX-3
10 proteins is upregulated under iron- or zinc- depleted conditions. Although it is
11 not clear precisely how ESX-3 effectors mediate iron uptake, they appear to
12 influence uptake of mycobactins, molecules that bind iron, and deliver it to the
13 mycobacterial cell [83]. ESX-3 was reported to be essential for growth in
14 pathogenic mycobacteria, although a recent study suggests that these
15 proteins are non-essential when iron is provided in an accessible form [85,
16 86]. However, some ESX-3 substrates may have iron-independent functions
17 in virulence [86]. It remains unclear whether type VII secretion plays a role in
18 metal uptake in non-mycobacterial systems.

19

20 ***Cell Envelope Integrity***

21 Recent work has indicated that the type VII effectors localise to the capsule of
22 the fish pathogen *Mycobacterium marinum* [87]. ESX-1 and ESX-5 effectors
23 also have an impact on the integrity of the mycobacterial capsule and the
24 stability of the cell wall [88, 89]. These studies suggest that T7SS has a key
25 function in bacterial cell envelope homeostasis, a fundamental requirement for

1 bacterial survival. Such cell envelope maintenance functions have thus far
2 been limited to mycobacteria.

3

4 ***Sporulation and Development***

5 The type VII system of the plant pathogen *Streptomyces scabies* does not
6 have a role in virulence, but rather surprisingly plays a role in development
7 and sporulation [90]. Defective spore formation has also observed in T7SS
8 effector mutants of the non-pathogenic *Streptomyces coelicolor* [91].

9 Interestingly, deletion of the ATPase (EccC) responsible for effector secretion
10 does not produce similar phenotypes, suggesting an intracellular role for
11 these effectors, perhaps in nucleoid restructuring, which could in turn
12 influence the developmental programme [90]. Twinned with the role of Esx
13 proteins in DNA transfer in mycobacteria, these observations suggest that
14 regulation of DNA interactions may be a recurrent theme in type VII secretion.
15 In *B. subtilis*, no sporulation defects have been reported for the *yuk/yue*
16 deletion mutants. However, YukE is controlled by phosphorylation of DegU, a
17 protein that accumulates in stationary phase and that has been associated
18 with cell differentiation in bacterial populations [57, 92].

19

20 ***Interbacterial Competition***

21 As described earlier, the EsaD nuclease produced by *S. aureus* serves to kill
22 competitor bacteria, while the cells producing the toxin produce antitoxins to
23 protect themselves [31]. Over half of the *S. aureus* strains analysed encode
24 EsaD, suggesting that it plays an important role in the ecology of this species.
25 In addition, the sequence of the nuclease domain is highly polymorphic,

1 consistent with a role in intra-species competition. *S. aureus* strains also
2 accumulate *esaG* homologues in strains that do not encode *esaD*, suggesting
3 a molecular arms race where strains accumulate anti-toxins to protect
4 themselves from polymorphic *EsaD* variants [31]. Along with recent evidence
5 from bioinformatics surveys, these observations suggest that interbacterial
6 competition represents a function common to many T7SSs. It also highlights
7 the parallels in versatility between type VI and type VII secretion, in that both
8 systems are capable of targeting eukaryotes and bacteria.

9

10 **Concluding Remarks**

11 A decade or more after its discovery, type VII secretion represents a
12 continuing, intriguing and important puzzle. Although there are meaningful
13 homologies visible in the T7SS apparatus and effectors across huge
14 phylogenetic distances, it has been hard to shoehorn their diverse features
15 into a simple one-size-fits-all functional framework. They target membranes,
16 proteins and DNA and have functions within and outside of the bacterial cell.
17 They govern interactions between bacteria and their conspecific competitors,
18 but also mediate host-pathogen interactions. They provide us with novel
19 vaccine and drug targets, as well as new tools for diagnosis. In closing, it is
20 worth stressing that much remains to be discovered (see Outstanding
21 Questions), as so far we have characterised just a few of these systems in the
22 laboratory. As bioinformatics surveys suggest, most of the vast landscape of
23 type VII remains uncharted and untraveled.

24

1 **Figure Legends**

2

3 **Figure 1. Distribution of WXG100 Domains, Key Components of Type VII**

4 **Secretion.** Genomic organization of the type VII secretion system clusters in
5 selected species representative of Actinobacteria: *M. tuberculosis* & *S.*
6 *coelicor*; Firmicutes: *S. aureus*, *L. monocytogenes*, *S. agalactiae*, *B. subtilis* &
7 *B. anthracis*; Gammaproteobacteria: *H. pylori*. All genetic loci contain a
8 member of the FtsK/SpoIIIE family (yellow) and at least one gene belonging to
9 the WXG100 superfamily (red). Shown in blue are genes associated with
10 toxin-antitoxin systems. Arrows represent direction of transcription and
11 relative length of the genes.

12

13 **Figure 2. The Staphylococcal Type VII Secretion System, Ess.** A model

14 showing the core Ess secretion machinery, substrates and accessory
15 proteins. Membrane-associated proteins (blue), substrates (yellow),
16 cytoplasmic proteins (green) and FtsK/SpoIII ATPase (red) are shown.

17

18 **Figure 3. Biological Functions of T7SS.** A model illustrating roles for T7SS

19 in bacterial cellular functions, intra/inter-bacterial interactions and host-
20 bacterial interactions.

Table 1. Distribution of WYG100 proteins in Diverse Bacteria^a

Phylum or Subphylum	Species
Bacteroidetes	<i>Bacteroides cellulosilyticus</i> , <i>Bacteroides intestinalis</i> , <i>Prevotella bryantii</i>
Chloroflexa	<i>Chloroflexus</i> sp MS-G, <i>Roseiflexus castenholzii</i>
Cyanobacteria	<i>Arthrospira</i> , <i>Arthrospira maxima</i> , <i>Arthrospira platensis</i> , <i>Arthrospira</i> sp. TJSD091, <i>Cyanobacterium aponinum</i> , <i>Geminocystis herdmannii</i> , <i>Leptolyngbya</i> sp. PCC 7376, <i>Lyngbya</i> sp. PCC 8106, <i>Pseudanabaena biceps</i>
Fusobacteria	<i>Fusobacterium periodonticum</i>
Lentisphaerae	<i>Lentisphaera araneosa</i>
Planctomycetes	<i>Gemmata obscuriglobus</i> , <i>Gemmata</i> sp. IIL30, <i>Gemmata</i> sp. SH-PL17, <i>Isosphaera pallida</i> , <i>Phycisphaera mikurensis</i> , <i>Phycisphaerae bacterium SM23_30</i> , <i>Pirellula</i> sp. SH-Sr6A, <i>Pirellula staleyi</i> , <i>Pirellula staleyi</i> DSM 6068, <i>Planctomyces</i> sp. SH-PL14, <i>Planctomyces</i> sp. SH-PL62, <i>Rhodopirellula baltica</i> WH47, <i>Rhodopirellula europaea</i> 6C, <i>Rhodopirellula islandica</i> , <i>Rhodopirellula maiorica</i> , <i>Rhodopirellula sallentina</i> , <i>Rhodopirellula</i> sp. SWK7, <i>Schlesneria paludicola</i> , <i>Singulisphaera acidiphila</i> , <i>Zavarzinella formosa</i>
α -Proteobacteria	<i>Acetobacter persici</i>
β -Proteobacteria	<i>Kingella denitrificans</i> , <i>Delftia acidovorans</i> , <i>Bordetella petrii</i>
δ -Proteobacteria	<i>Desulfatibacillum aliphaticivorans</i>
ϵ -Proteobacteria	<i>Helicobacter bizzozeronii</i> , <i>Helicobacter felis</i> , <i>Helicobacter heilmannii</i> , <i>Helicobacter trogonum</i> , <i>Sulfurimonas gotlandica</i>
γ -Proteobacteria	<i>Aeromonas bestiarum</i> , <i>Alcanivorax pacificus</i> , <i>Halomonas hydrothermalis</i> , <i>Methylobacterium methanica</i> , <i>Pseudomonas aeruginosa</i> , <i>Pseudomonas lundensis</i> , <i>Pseudomonas stutzeri</i> , <i>Pseudomonas taeanensis</i> , <i>Pseudomonas veronii</i> , <i>Stenotrophomonas maltophilia</i> , <i>Vibrio cholerae</i> , <i>Vibrio parvulus</i>

^aThis tabulation draws on the taxonomic distribution of the WYG100 PFAM domain PF06013 and on PSI-BLAST searches of the NCBI non-redundant sequence databases performed with the EsxA and Hp0062 sequences from *M. tuberculosis* and *H. pylori*.

Table 2. Summary of Type VII Effectors and Their Functions^a

Type VII Effector^a	Species	Roles
EsxA	<i>M. tuberculosis</i> <i>M. marinum</i>	Cell membrane lysis [2], activation of interferon responses [69], T-cell activation [76], induction of cell apoptosis [70], virulence [3, 4]
	<i>M. smegmatis</i>	DNA transfer [80]
	<i>S. scabies</i> <i>S. coelicolor</i>	Spore formation [90, 91], cell development [91], phage resistance [90]
	<i>S. aureus</i>	Modulation of host cell apoptosis [39], virulence [41, 43], protective antibody response [79]
	<i>L. monocytogenes</i>	Unknown
EsxB	<i>M. tuberculosis</i> <i>M. smegmatis</i>	Cell membrane lysis [2], virulence[3]
	<i>S. scabies</i> <i>S. coelicolor</i>	Spore formation [90, 91], cell development [91], phage resistance [90]
	<i>S. aureus</i>	Virulence [43], protective antibody response [79]
	<i>B. anthracis</i>	Unknown
EsxG	<i>M. tuberculosis</i>	Iron and zinc uptake [83, 84]
	<i>M. smegmatis</i>	Iron uptake [83]
EsxH	<i>M. tuberculosis</i>	Iron and zinc uptake [83], intracellular growth, phagosome maturation [93] T-cell immunogen
	<i>M. smegmatis</i>	Iron uptake [83]
EspA	<i>M. tuberculosis</i>	Virulence, cell wall integrity* [88]
PE19	<i>M. tuberculosis</i>	Virulence, cell wall permeability [77, 94]
PE5	<i>M. tuberculosis</i>	Iron uptake
PPE4	<i>M. tuberculosis</i>	Iron uptake
PPE-10	<i>M. marinum</i>	Capsule integrity [89]
EsaD	<i>S. aureus</i>	Interbacterial competition [31]
EsxC	<i>S. aureus</i>	Bacterial persistence [43]
EsxD	<i>S. aureus</i>	Unknown
YukE	<i>B. subtilis</i>	Unknown
EsxP	<i>B. anthracis</i>	Unknown
EsxW	<i>B. anthracis</i>	Unknown

^a Mycobacterial substrates EsxM, EsxN, EspB, EspC, EspE, EspF, EspJ, EspK, PE15, PPE20, PE35, PPE68, PE25 and PPE41, all of unknown function, have not been included in this table. * EspA does not play a role in cell wall integrity in all *M. tuberculosis* strains [95]

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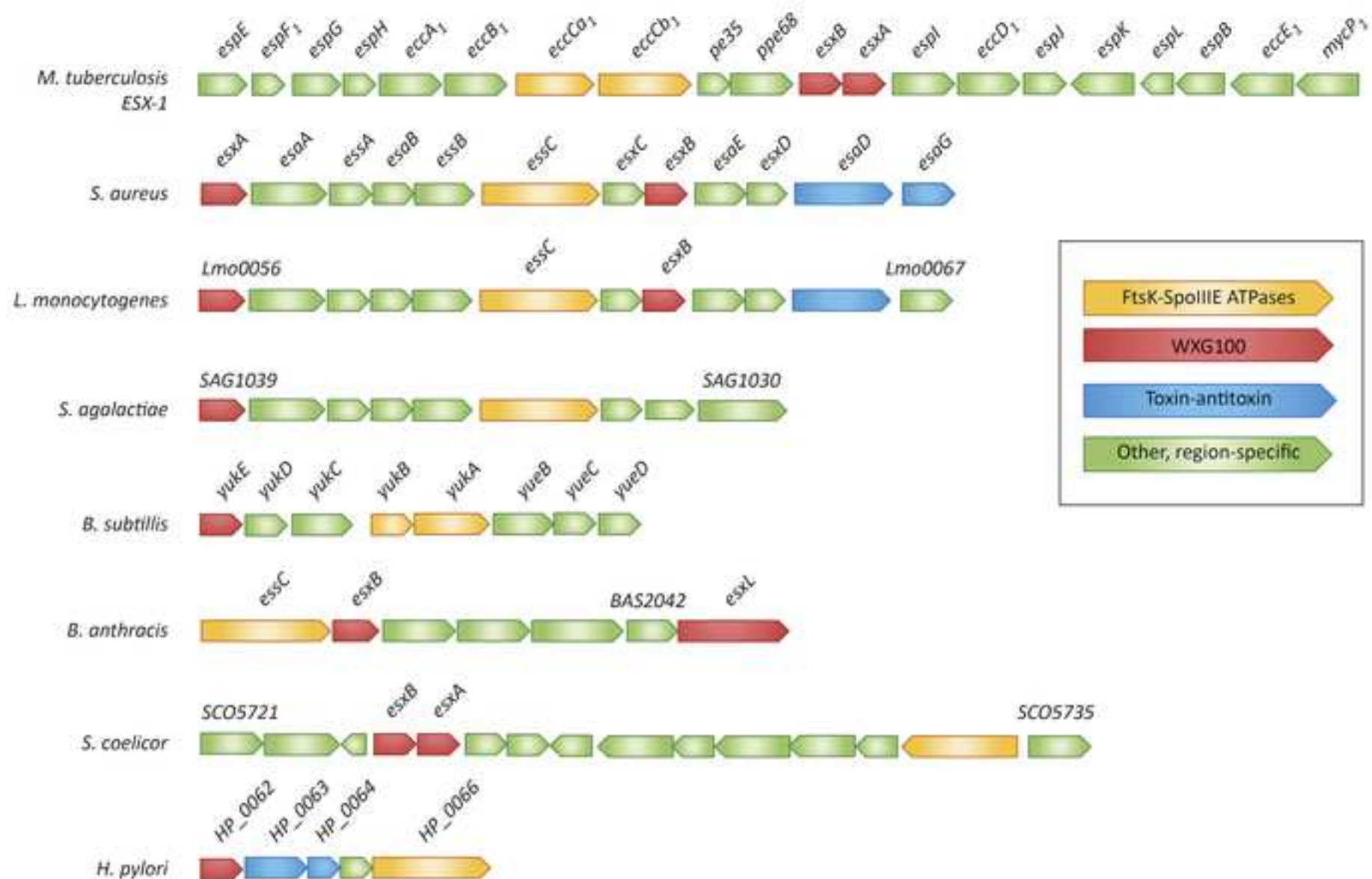
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Figure 1

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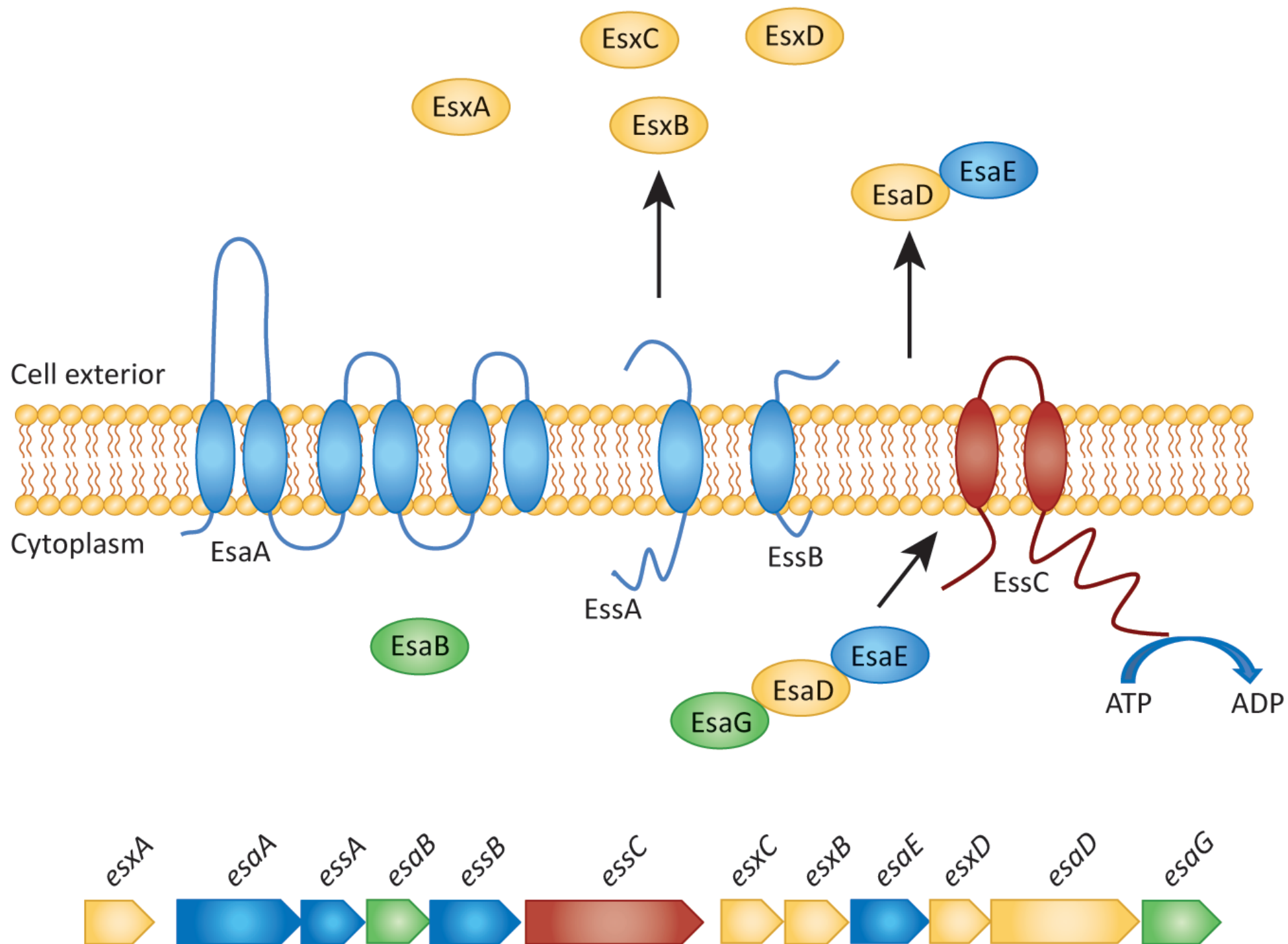
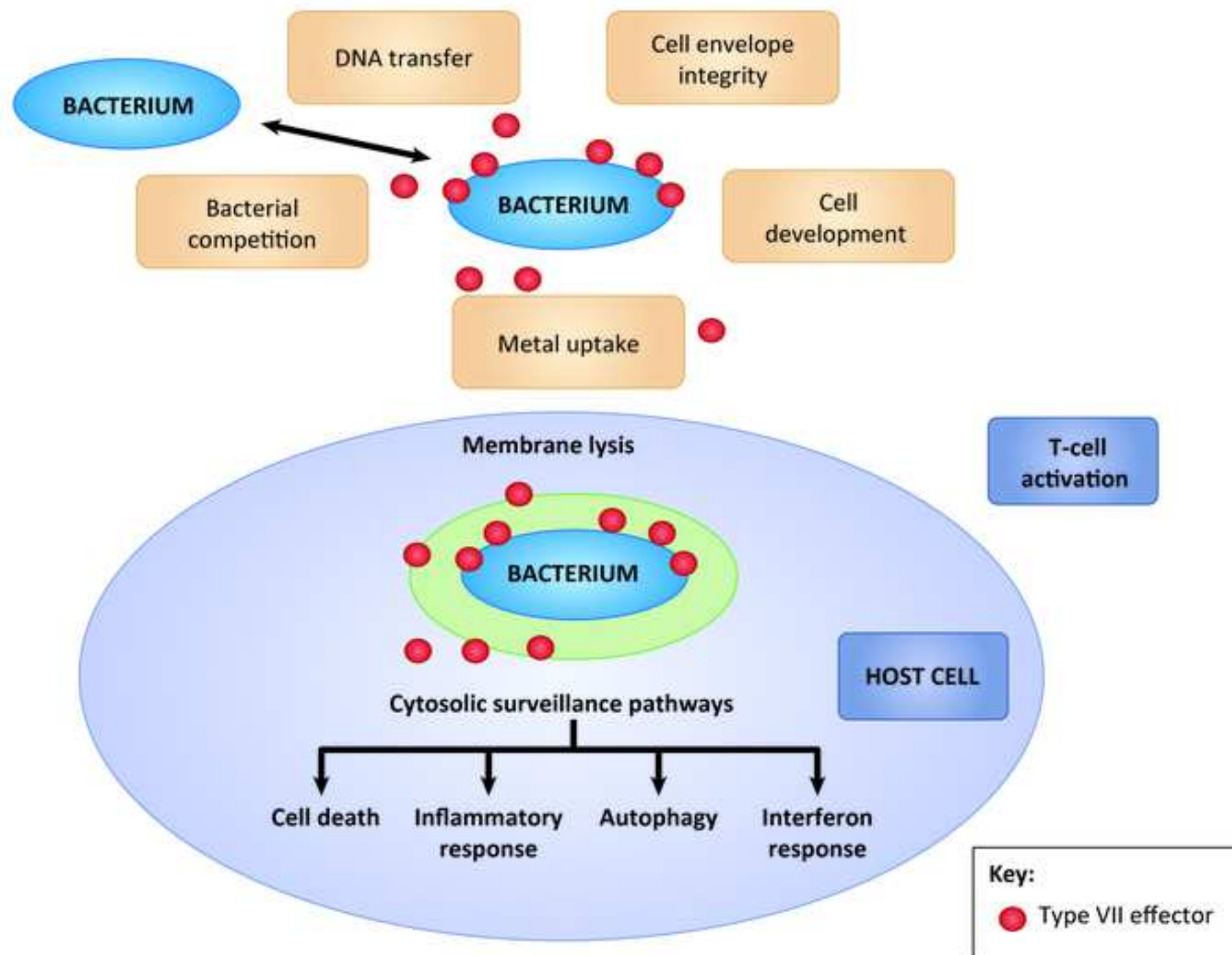


Figure 3



Trends

- T7SSs are more widespread among bacteria than previously thought; unlike other bacterial secretion systems, they are present across diverse bacterial phyla, in both Gram-positive and Gram-negative bacteria.
- Non-mycobacterial T7SSs have distinct secretion machinery and mechanisms of secretion compared to their mycobacterial counterparts.
- Bioinformatics surveys have highlighted an association between toxin-antitoxin modules and type VII secretion in a number of bacterial species and a recent report provides experimental evidence of a role in intra-bacterial interactions in *S. aureus*.
- Type VII effectors display a range of species-specific cellular functions, and can modulate interbacterial interactions as well as interactions between bacteria and eukaryotic cells.

Outstanding Questions

1. What is the structure of the T7SS apparatus?
2. What role does the T7SS play in Gram-negative bacteria? Are substrates targeted to the periplasm or are they exported from the cell? If they leave the cell, how do they cross the outer membrane?
3. Is there crosstalk between T7SSs and other secretion systems?
4. Are there common functions for the T7SSs across pathogenic and environmental bacteria?
5. How do the Esx toxin-antitoxin modules influence interactions within bacterial communities in the environment or during colonisation and infection?
6. Which host proteins do Esx proteins interact with during intracellular infection?
7. What is the role of staphylococcal T7SS effectors during persistent infection?